A VOLATILE TRAIL PHEROMONE OF THE LEAF-CUTTING ANT, *ATTA TEXANA*

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Abstract—The major volatile trail-marking pheromone of the Texas leaf-cutting ant, *Atta texana*, was isolated, identified as methyl 4-methylpyrrole-2-carboxylate, and synthesized. The synthesized pheromone elicited strong trail-following responses from workers in the laboratory and the field.

INTRODUCTION

SOCIAL insects have a very sophisticated chemical communications system that controls food collection and nest activities. This phenomenon has been studied extensively and the literature has been thoroughly reviewed by WILSON (1965a, b), BLUM (1969), and MOSER (1970). In this complex olfactory system, one of the most highly developed pheromone triggered responses is the trail following-behaviour of ants and termites. The trail pheromones of termites seem to be non-specific within genera (STUART, 1963; SMYTHE *et al.*, 1967) and it is not known in all cases whether these trail-marking compounds are synthesized by the insects or obtained by ingestion. In one instance the trail pheromone of the termite, *Reticulitermes virginicus*, identified as *cis*-3-*cis*-6-*trans*-8-dodecatrien-1-ol, was obtained from fungus-infected wood as well as from the termites (MATSUMURA *et al.*, 1968). The only other trail pheromone identified chemically is hexanoic acid, isolated from the termite *Zootermopsis nevadensis* Hagen (HUMMEL and KARLSON, 1968).

Many species of ants are known to use trail pheromones, and trail sharing has been reported in some species (WILSON, 1965a). The trail-following behaviour of the Texas leaf-cutting ant or town ant, *Atta texana* (Buckley), was described by MOSER and BLUM (1963). MOSER and SILVERSTEIN (1967) found that the trail pheromone of the town ant contains at least two components, one volatile and the other non-volatile. In a brief, preliminary communication, TUMLINSON *et al.* (1971) reported that methyl 4-methylpyrrole-2-carboxylate was the major volatile component of the trail pheromone of *A. texana*.

We now describe the isolation of this compound from *A. texana*, its identification and synthesis, and the behaviour it elicits in laboratory and field colonies.

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MATERIALS AND METHODS

Worker ants of mixed sizes were collected from nests in Grant Parish, Louisiana. The whole bodies (3·7 kg) of workers were macerated in methylene chloride and the soluble material was distilled in a short-path still at 90°C and 0·05 mm Hg onto a dry ice cooled condenser.

The biologically active distillate was further fractionated by gas–liquid chromatography (GLC). Column A, 4% SE-30 on 45/60 mesh Chromosorb G, 1 m × 8 mm (i.d.), 50 cm³/min He flow, was held at 55°C for 4 min after injection, then programmed to 100°C at 25°/min, held at 100°C for 6 min, then programmed to 200°C at 30°/min, and held at 200°C for the remainder of the run. This column was connected to a four-port backflush valve so that the flow through the column could be reversed. At 55 min after injection the column was backflushed for 55 min. The remaining columns were operated in the normal manner as follows: (B) 4% diethylene glycol succinate on 60/80 mesh Gas Chrom Q, 2 m × 4 mm, 20 cm³/min He flow, 142°C isothermal; (C) 4% Carbowax 20M on 60/80 Chromosorb G, 7·3 m × 2·4 mm, 25 cm³/min He, 170°C isothermal; (D) 10% silicone DC QF-1 on 60/80 Chromosorb G, 3 m × 2·4 mm, 20 cm³/min He, 130°C isothermal.

Fractions collected from the chromatograph by condensing the column effluent in a 12 in. glass capillary tube held in a thermal gradient collector (BROWNLEE and SILVERSTEIN, 1968) were bioassayed (see later) and the active fractions were then chromatographed on the succeeding column.

Spectral analyses of the synthesized and natural pheromones were performed on gas chromatographically pure samples. Infra-red spectra were obtained with about 50 μg of pheromone dissolved in 10 μl of carbon tetrachloride contained in a Barnes–Engineering micro-cavity cell. The micro-cell was mounted in the condensed beam of a Perkin–Elmer Model 621 i.r. spectrophotometer. Good NMR spectra resulted from 25 time-averaged scans, in a Varian HA-100 spectrometer, of 125 μg of pheromone dissolved in carbon tetrachloride containing tetramethylsilane as an internal standard.

For a low-resolution mass spectrum 5 μg of the compound, collected from the gas chromatograph and sealed in a glass capillary tube, was introduced into the micro-inlet system of a Hitachi RMU-6 mass spectrometer. For the high-resolution spectrum, 25 μg was introduced into a CEC, Model 21-110B, mass spectrometer in a similar manner. The mass values were measured by the peak-matching technique. Plate spectra and a high-resolution oscillographic chart scan were also recorded.

Synthesis of the pheromone was accomplished by distilling diazomethane, prepared from N-methyl-N-nitroso-p-toluenesulfonamide (HOPPS, 1970), into an ethereal solution of 20 mg of 4-methylpyrrole-2-carboxylic acid. The excess diazomethane and the ether were removed by evaporation and the product was purified by GLC on column B.

The synthesized pheromone and all fractions resulting from each step of the isolation procedure were tested by the laboratory assay of MOSER and BLUM (1963).

An amount of each fraction equivalent to about 2 g of whole ants was dissolved in 50 μl of chloroform giving a concentration of 40 mg equivalents of ants/μl. A portion of this solution was then diluted to give solutions of 4 mg/μl and 0·4 mg/μl. Every fraction was tested for activity at each of these concentrations. The synthesized pheromone (2 mg) was dissolved in 0·5 ml of chloroform giving a concentration of 4 μg/μl. This solution was serially diluted, by factors of 10, to 0·4 pg/μl. All eight of these concentrations were tested for activity. Circles 50 cm in circumference were described on slick cardboard sheets with 10 μl of each sample. Fifteen minor workers from a laboratory colony were then released into the centre of the circle. Only the first 10 that followed the artificial trail at least 15 cm were counted. Response was considered weak when 3 or 4 workers followed the trail, medium if 5, 6, or 7 followed, and strong if 8 to 10 workers responded.

Field tests were conducted with the synthesized pheromone by erasing portions of a natural trail and replacing the erased portion with an artificial trail laid on a strip of cardboard. In another test the pheromone in chloroform solution was dribbled directly onto the sand leading away from the natural trail at a 45° angle.

RESULTS AND DISCUSSION

As reported previously (MOSER and SILVERSTEIN, 1967) the non-volatile residue from the short-path distillation evoked trail-following response by minor workers. This material was saved for further study. The distillate from the short-path distillation was very active in the laboratory assay and was fractionated by GLC without any intermediate purification.

When the crude distillate was chromatographed on column A, the first fraction, consisting mostly of solvent, elicited slight trail following when the equivalent of 2 g of whole ants was streaked on the circle. The second fraction, collected between 12 and 28 min, elicited strong trail following when 4 mg equivalents was used to describe the circle. The third fraction, collected from 28 to 55 min, was about one-tenth as active as the second, and the higher boiling materials, collected by reversing the flow and backflushing the column for 55 min, were also slightly active. Large injections (1 ml) were made on this column, but since the distillate consisted mostly of solvent, the solute did not appear to overload the column. Great care was taken to avoid cross-contamination of fractions. Therefore it appears that the wide range of activity can be attributed to the presence of more than one active compound. Additionally, subsequent chromatography of these four fractions showed that the active materials in the various fractions eluted at different times from other columns.

The second fraction from column A was then chromatographed on column B. The material eluted between 27·5 and 35·5 min was very active when 4 mg equivalents were streaked on the circle. When the active material from column B was chromatographed on column C a major component collected between 49 and 54 min elicited strong trail following at the 0·4 mg equivalent level. Further purification on column D yielded one major component collected from 13·2 to 15·3 min and two minor components amounting to only 5 and 1 per cent of the

major compound which were collected together in a fraction eluted between 40 and 72 min. The major component elicited trail-following response from 100 per cent of the minor workers at the 4 mg equivalent level and the mixture of the two minor components elicited weak response at the 40 mg equivalent level. None of the other fractions collected from column D, including that collected between the two active fractions, produced the slightest response at less than 400 mg equivalents.

About 150 μg of the major active compound was collected from column D. Rechromatography of this compound on all four columns gave only one peak in each case indicating that it was homogeneous. The average worker collected in the field weighs about 15·4 mg. Thus, ignoring losses incurred in processing, we estimate that each worker contains about 0·6 ng of this compound.

The low-resolution mass spectrum and the i.r. spectrum of this compound are shown in Fig. 1. The parent ion at 139 m/e in the low-resolution mass spectrum was found to have a mass of 139·0640 by high resolution mass spectroscopy, definitive for the molecular formula $C_7H_9O_2N$. The mass values of the other major fragments, measured by high-resolution mass spectroscopy, were in agreement with those expected for a substituted pyrrole carboxylate. The i.r. spectrum shows N–H stretching bands at 3465 cm^{-1} and 3320 cm^{-1} and a characteristic carbonyl absorption at 1695 cm^{-1}. In the NMR spectrum a three-proton singlet at 7·86τ and one at 6·16τ are characteristic of a methyl attached to an aromatic or heteroaromatic ring and a methyl attached to the oxygen in a methyl ester, respectively. One-proton overlapping multiplets at 3·32τ and at 3·38τ can be assigned to the ring protons. A barely detectable broad signal from 1·2τ to 0·4τ confirms the N–H moiety. Methyl 4-methylpyrrole-2-carboxylate (I) has the structure most consistent with the spectral data.
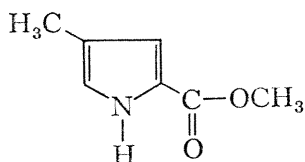
I

Compound I has been previously synthesized (Rapoport and Bordner, 1964) and we obtained 20 mg by esterification of 4-methylpyrrole-2-carboxylic acid. The mass, i.r., and NMR spectra of the synthesized compound were congruent with those of the natural product. Additionally, GLC retention times of the natural and synthesized pheromone were identical on all four columns. More recently, compound I was synthesized by Sonnet (1971) and this material was identical chemically and in biological activity to our synthetic product.

When synthesized I was assayed in the laboratory, the lower threshold of detection for minor workers was 0·08 pg/cm ($3·48 \times 10^8$ molecules/cm). Strong responses were obtained from 0·8, 8·0, and 80·0 pg/cm. The pheromone obtained

by isolation evoked strong responses at about the same levels. Some repellency was evident with the synthesized pheromone at 0·8 and 8·0 ng/cm, and greater concentrations strongly repelled minor workers.
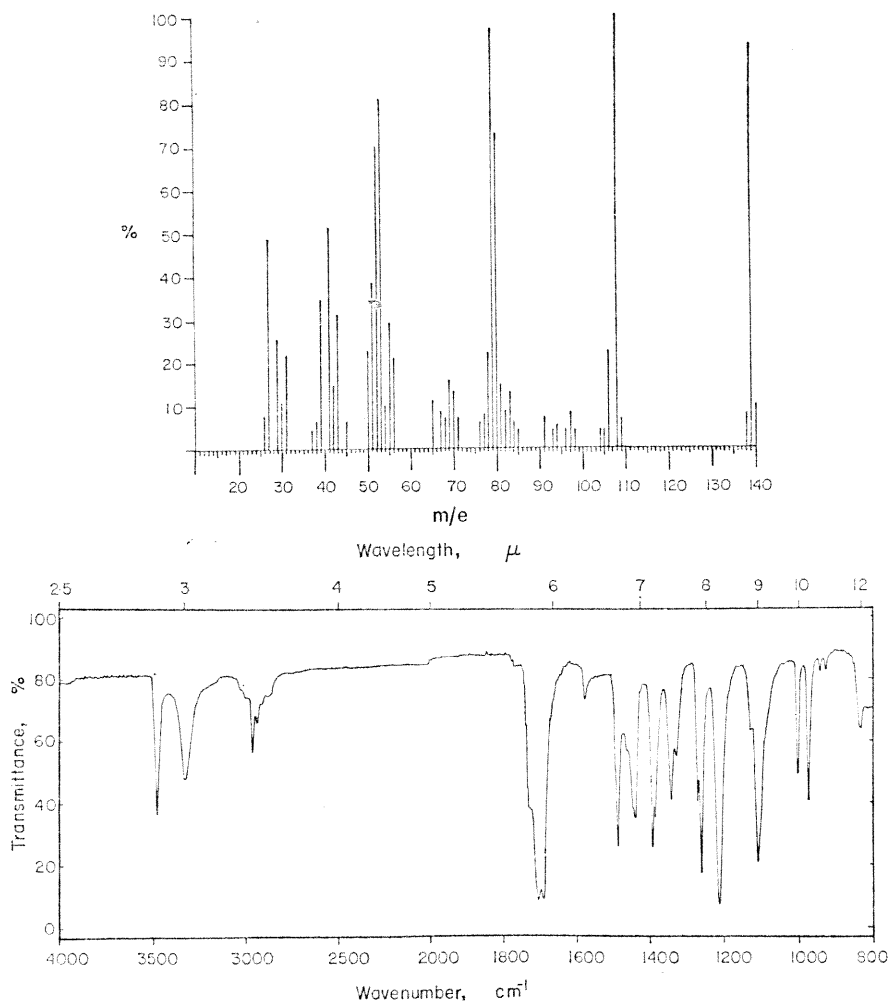

FIG. 1. Mass and i.r. spectra of methyl 4-methylpyrrole-2-carboxylate.

In the field, medium- and large-sized workers readily followed trails produced with the synthesized pheromone. Erasing portions of a natural trail leading from a nest disrupted the worker trail-following activity. When the erased portion was bridged with a 2·7 ng/cm trail of synthesized pheromone on a 15 cm cardboard strip the workers resumed following this trail. They detected another trail made by dribbling a 4·0 pg/μl solution on the sand at a 45° angle to the field trail; response was strong at 40·0 pg/μl.

The results of this study prove conclusively that methyl 4-methylpyrrole-2-carboxylate is the major volatile trail pheromone of *A. texana*. The function of the other unidentified volatiles that elicit trail-following response is unknown. Recently SONNET and MOSER (1972) have demonstrated that *A. texana* workers respond to several analogues of methyl 4-methylpyrrole-2-carboxylate. Also, MOSER *et al.* (in preparation) found that compound I was equally potent as a trail marker for several attines from the most primitive to the most advanced species, but that species from other subfamilies failed to respond. We might speculate that other active components of the *Atta* trail pheromone are identical to some of the synthesized analogues and that they have other functions in the communication system, or serve to distinguish *A. texana* trails from those of other closely related species.

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